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Abstract

Aeromonas are water-borne pathogens. They are halotolerant, which means that they can survive in environments whose salt content corresponds to that of seawater (3.0% NaCl). However, the presence of Aeromonas in seawater is extremely rare compared with that in river water. In this study, we tested the ability of Aeromonas sobria to produce toxins in river water and seawater. First, we cultured A. sobria on skim milk agar plates supplemented with either river water (SARW) or seawater (SASW). The bacteria grew on both plates. A clear zone around the bacteria was generated in SARW. However, such a zone was not observed in SASW, suggesting that proteases were not generated in SASW. Subsequently, we cultured A. sobria in a nutrient broth supplemented with either river water (NRW) or with seawater (NSW), and examined the protease activity of their culture supernatants. The protease activity of the culture supernatant from NSW was extremely low compared to that from NRW. The immunoblotting analysis showed that serine protease (ASP) was not produced by the culture in NSW. By contrast, aerolysin-like hemolysin was produced in all conditions examined in this study. This indicates that the salinity of water is deeply involved in the production of ASP by A. sobria.

KEYWORDS: Aeromonas, water, toxin, salinity

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Original Article

Toxin Production by *Aeromonas sobria* in Natural Environments: River Water vs. Seawater

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Aeromonas **are water-borne pathogens. They are halotolerant, which means that they can survive in environments whose salt content corresponds to that of seawater (3.0% NaCl). However, the presence of *Aeromonas* in seawater is extremely rare compared with that in river water. In this study, we tested the ability of *Aeromonas sobria* to produce toxins in river water and seawater. First, we cultured *A. sobria* on skim milk agar plates supplemented with either river water (SARW) or seawater (SASW). The bacteria grew on both plates. A clear zone around the bacteria was generated in SARW. However, such a zone was not observed in SASW, suggesting that proteases were not generated in SASW. Subsequently, we cultured *A. sobria* in a nutrient broth supplemented with either river water (NRW) or with seawater (NSW), and examined the protease activity of their culture supernatants. The protease activity of the culture supernatant from NSW was extremely low compared to that from NRW. The immunoblotting analysis showed that serine protease (ASP) was not produced by the culture in NSW. By contrast, aerolysin-like hemolysin was produced in all conditions examined in this study. This indicates that the salinity of water is deeply involved in the production of ASP by *A. sobria*.**

Key words: *Aeromonas*, water, toxin, salinity

Aeromonas species have been implicated repeatedly as the causative agents of clinical illnesses, often serious, ranging from gastrointestinal and wound infections to septicemia [1-3]. There are 16 species in *Aeromonas* and 3 species, *A. sobria*, *A. hydrophila*, and *A. caviae*, represent more than 85% of the clinical isolates [1, 4]. Among these, *A. sobria* is reported to the predominant pathogen in humans [3].

Aeromonas have been isolated from a wide variety of aquatic environments including fresh, brackish, and marine water [5-9]. *Aeromonas* can grow in the presence of 3.0% NaCl whose salt concentration is almost equivalent to that of seawater. Therefore, *Aeromonas* have been considered halotolerant [10, 11]. However, they tend to prefer fresh water more than marine water [6, 7]. There is increasing evidence that *Aeromonas* infections in human are closely related to water exposure [12, 13]. In particular,

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the incidence of patients with *Aeromonas* is high among those who suffer trauma while submerged in freshwater. On the other hand, infection from seawater injuries is uncommon [3, 14–18].

Aerolysin-like hemolysin (ALH) and proteases are well known as the exotoxins of *Aeromonas*. ALH has been shown to possess 2 kinds of toxicity [19]. One is cytotoxicity and the other is enterotoxicity. ALH is cytotoxic to mammalian cells by making small pores in their membranes; hence, cells that are attacked by ALH are ruptured [19]. The enterotoxic activity of ALH has been clearly demonstrated by intestinal loop assay; however, the mechanism has remained unclear. We observed that ALH stimulates the production of prostaglandin E_2 via cyclooxygenase-2 in intestinal cells [20]. However, the contribution of this stimulation to the diarrhea caused by ALH has not been determined.

Two proteases, serine protease (ASP) and metalloprotease (AMP), are reported as extracellular proteases produced by *Aeromonas* [21, 22]. The action of ASP has been well studied. An experiment using purified ASP showed that ASP induces vascular leakage and blood pressure lowering through activation of the kallikrein-kinin system [23]. Moreover, it was shown that ASP promotes human plasma coagulation via activation of human prothrombin. Furthermore, it was shown that platelet aggregation is induced by ASP-treated prothrombin. Thus, ASP-mediated a-thrombin production likely contributes to the induction of disseminated intravascular coagulation (DIC), which is a common and deadly consequence in sepsis patients [24]. In contrast, the action of AMP has remained unclear.

It has been considered that bacteria produce exotoxins to survive in undesirable conditions. For example, bacterial exotoxins destroy the tissue of the host and weaken the killing action of the host. Simultaneously, the bacteria can utilize the destroyed materials from the cell as nutrition. [25–27]. Therefore, it is a matter of life and death for bacteria to produce exotoxins in the condition of infection. As described, the number of *Aeromonas* in seawater is low compared with that in river water, though *Aeromonas* itself is halotolerant. In recent years, a number of reports about the survival ability of this pathogen in various conditions have been published [7, 28, 29]. However, little is known about the production of toxins by *Aeromonas* in different types of environmental water. In this study, we examined the productions of ASP and ALH in river water and seawater.

Materials and Methods

Bacterial strains. A stock culture of *A. sobria* 105 was primarily investigated in this study. This strain was previously isolated from a well water reservoir in Japan and used as environmental isolate in this study. Other strains used in this study are listed in Table 1. These strains were isolated from patients with diarrhea in Japan (n=6) and Bangladesh (n=2).

Water samples. River water from Asahi

Table 1 Proteolytic and hemolytic activities of *A. sobria* on solid media supplemented with river water or seawater

Lab ID #	Source (country isolated)	Protease activity on skim milk agar		Hemolytic activity on blood agar		
		NARW[a]	NASW[b]	NARW	NASW	NASW plus trypsin[c]
105	Environment (JP)	+	−	+[d]	−[e]	+
288	Clinical (JP)	+	−	+	−	+
102	Clinical (JP)	+	−	+	−	+
106	Clinical (JP)	+	−	+	−	+
108	Clinical (JP)	+	−	+	−	+
109	Clinical (JP)	+	−	+	−	+
111	Clinical (JP)	+	−	+	−	+
118	Clinical (BD)	+	−	+	−	+
119	Clinical (BD)	+	−	+	−	+

[a]NARW, agar plate prepared with river water; [b]NASW, agar plate prepared with seawater; [c]NASW plus trypsin, trypsin solution was poured into hole made next to the bacteria after bacteria were cultured on a seawater agar plate; [d]+, existence of a transparent zone after cultivation at 37℃ for 20h; [e]−, lack of a transparent zone after cultivation at 37℃ for 20h; JP, Japan; BD, Bangladesh.

River, Okayama and seawater from the coastal zone of Honjima Island in Seto Inland Sea of Japan were collected in sterile containers. The pHs of the river water and seawater were 6.93 and 8.23, respectively. The salinities of the river water and seawater were estimated to be $<0.2\%$ (conductivity, 102 μS/cm at 23.8℃) and 3.1% (conductivity, 46,100 μS/cm at 23.8℃), respectively. The water samples were sterilized by membrane filtration. The sterility of the filtered water was confirmed by plating on nutrient agar medium. No colonies from the filtered water were found on agar medium after incubation at 37℃ for 48 h. Unless otherwise stated, all the river water or seawater samples used in this study were filter sterilized.

Media preparation. Nutrient liquid medium-supplemented water (50 ml) was prepared in 300 ml side-arm conical flasks with river water or seawater. First, 0.54 g of powder of nutrient broth (Eiken Chemical Co., Tokyo, Japan) was dissolved in 10 ml river water or seawater, and autoclaved at 121℃ for 15 min. This amount of powder corresponds to the amount required to make 30 ml of nutrient broth in the usual preparation, according to the manufacturer's instructions. Then, 40 ml river water or seawater was added to the corresponding flasks aseptically. The prepared liquid media supplemented with river water and seawater are designated NRW and NSW, respectively. Final salt concentrations in NRW and NSW were approximately 0.3% and 3.4%, respectively.

Skim agar milk agar plates (final concentration of skim milk in medium is 3%) and sheep blood agar plates (final concentration of sheep blood cells in medium is 5%), which were supplemented with river water and sea water, were prepared as follows using nutrient agar medium (Eiken Chemical Co.) as the basal medium.

To prepare skim milk agar plates, 1.75 g of the nutrient agar medium powder (corresponding to the amount required to make 50 ml of nutrient agar medium in the usual preparation) was dissolved in 25 ml river water or seawater, and autoclaved at 121℃ for 15 min. Then 1.5 g of skim milk dissolved in 25 ml river water or sea water was autoclaved at 121℃ for 15 min. The 2 solutions, nutrient agar medium solution and skim milk solution, were mixed at 50℃ aseptically and poured into petri dishes. The plates thus prepared were designated skim milk agar plate of NARW (nutrient agar supplemented with river water) and skim milk agar plate of NASW (nutrient agar supplemented with seawater).

To prepare sheep blood agar plates (5%), 1.75 g of the nutrient agar medium powder was dissolved in 47.5 ml of river water or seawater. To the medium dissolved with river water, 0.19 g of NaCl was added to reach an NaCl concentration of 0.9%. These media were autoclaved at 121℃ for 15 min. Sheep blood cells (2.5 ml) were added to the medium at 50℃ aseptically, and the mixture was poured into petri dishes at 50℃ aseptically. The plates thus prepared were designated blood agar plates of NARW (supplemented with river water) and blood agar plates of NASW (supplemented with seawater).

Bacterial growth, protease activity, and hemolytic activity. Growth of bacteria in liquid medium was determined by measuring the optical densities at 620 nm of the culture.

Protease activity and hemolytic activity of bacteria on solid media were determined by culturing the bacteria on skim milk agar plates and blood agar plates, respectively. After cultivation, the activity was judged by the appearance of a transparent zone around the bacteria. Strains producing a transparent zone were judged to be positive in the reaction.

The proteolytic activity of the culture supernatant was determined as previously described [21]. Briefly, a portion (50 μl) of the sample was added to a reaction mixture containing 0.4% azocasein in 10 mM Tris-HCl buffer (pH 7.5) to a final volume of 500 μl. The mixture was incubated at 37℃ for 1 h, and the reaction was stopped by adding 500 μl of 10% trichloroacetic acid (TCA). After centrifugation at 13,000 × g, 500 μl of the supernatant was mixed with an equal volume of 1 N NaOH and the optical densities of the solutions were measured at 450 nm.

The hemolytic activity of the culture supernatant was determined by the tube method as previously described [19]. Briefly, sample aliquots of 0.75 ml were serially two-fold diluted with 10 mM Tris-HCl buffer (pH 7.2) containing 0.9% NaCl and mixed with an equal volume of a suspension containing washed sheep erythrocytes at a ratio of 2% in the buffer. After incubation at 37℃ for 1 h, the mixture was centrifuged at 1,000 × g for 5 min at 4℃. The supernatants were removed and their optical densities at 540 nm were read. One hemolytic unit was defined as

the amount of toxin causing 50% hemolysis under these conditions. The hemolytic activity of the samples was expressed by the reciprocal of the dilution of the samples to give one unit.

The trypsinization of the culture supernatant was carried out as follows. A solution containing trypsin (10 mg/ml in 10 mM Tris-HCl buffer, pH 7.2) was added to the culture supernatant at a ratio of 1% per volume, and then incubated at 37℃ for 10 min. The hemolytic activity of the mixture was examined as described above.

Detection of ALH and ASP by immunoblotting. Proteins in the culture supernatant of *A. sobria* 105 were precipitated by treatment with TCA. TCA solution was added to 1.0 ml of culture supernatant to reach the concentration of 10.0%. The mixture was allowed to sit for 30 min at room temperature and the precipitates yielded were collected by centrifugation. After rinsing with ethanol, the precipitates were solubilized with 100 μl of loading solution for SDS-PAGE, and a portion (15 μl) of the sample was loaded onto a lane of SDS-polyacrylamide gel containing 16% acrylamide. After electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membranes and detected using antiserum against ALH and ASP [30], and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences, UK Limited, Buckinghamshire, UK) according to the manufacturer's instructions.

Statistical analysis. Results were expressed as the means ± standard deviations of 2 experiments performed in triplicate. Statistical analysis was performed with Microsoft Excel. A two-tailed Student's t test was considered significant if p values were < 0.001. Data with p values presenting significant differences are indicated by a single asterisk.

Results

Protease and hemolytic activities on solid medium. All isolates were inoculated onto skim milk agar plates and blood agar plates supplemented with sea water and river water and incubated at 37℃ for 20 h. All isolates grew under these conditions. The transparent zone on skim milk agar plates was generated by the action of extracellular proteases from bacteria, which degraded the insoluble casein in the medium into soluble peptides. The transparent zone on the blood agar plates was generates by the action of extracellular hemolysin, which degraded the sheep erythrocytes in the medium.

A transparent zone appeared around the streaking lines of all isolates examined on both skim milk agar medium and blood agar medium supplemented with river water (Table 1). But in both types of plate supplemented with sea water, no transparent zone appeared, even though the growth of bacteria was observed.

As described later, ALH is released outside of the cell as an inactive precursor form. The precursor is converted into the active form by undergoing cleavage with proteases such as trypsin. We made a small hole near the bacteria on the blood agar plate containing seawater, poured trypsin solution (20 μl, 10 mg/ml) into it, and incubated the plate at 37℃ for 2 h. By this treatment, the precursor was processed to the active form and the transparent zone appeared in all isolates. This showed that hemolysin was produced by *Aeromonas* grown on agar medium containing seawater, and that, without trypsin treatment, the hemolysin produced remained in the precursor form because of the lack of proteases from *Aeromonas*.

Production of hemolysin and serine protease in liquid medium supplemented with river water and seawater. In the above experiment, we used solid media to examine the ability of *A. sobria* to produce toxins. Subsequently, in order to clarify the universal validity of the effect of different environmental waters, we examined the growth and the ability of the bacteria to produce toxins in liquid media. Two strains, one from the environment (*A. sobria* 105) and another from a patient (*A. sobria* 288), were used in this examination. Both strains were cultured in NRW and NSW at 37℃ with shaking (140 rpm), and the growth of bacteria was monitored. The bacteria grew much better and faster in NRW than in NSW; however, it was shown from this experiment that both strains of *Aeromonas* could grow in NSW (Fig. 1).

The culture supernatants were prepared from 12-h bacterial suspensions. Serine protease in the culture supernatants was detected by Western blotting using anti-ASP antiserum [21]. The band for ASP was detected in lanes for the samples from NRW of both strains, *A. sobria* 105 and *A. sobria* 288; however, the

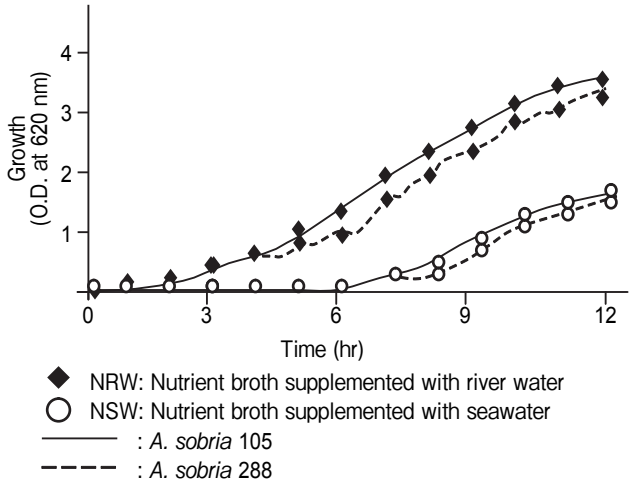


Fig. 1. Growth of *A. sobria* 105 and *A. sobria* 288 in nutrient broth supplemented with river water or seawater. *A. sobria* strains were cultured in nutrient broth supplemented with river water (NRW) or seawater (NSW) at 37℃ with shaking (140 rpm). Bacterial growth was monitored by the absorbance at 620 nm.

band was not detected in the lane for samples from NSW of both strains (Fig. 2, gel 1), indicating that the production of ASP was suppressed when *A. sobria* was cultured in NSW.

The production of ALH in liquid medium was examined, too. It has been shown that ALH emerges as a precursor in culture medium and then the precursor is processed to the active form by protease(s) released by the bacteria [30]. In *in vitro* experiments, ALH can be processed by incubation with trypsin, as shown in Table 1.

Two bands for ALH appeared in both samples, prepared from cultures in media supplemented with river water (NRW) and seawater (NSW) in both strains (Fig. 2, gel 2). One band is the precursor form and the other is the active form. In the sample from NSW, the density of the band of the active form was weaker than that of the precursor form. In contrast, in the sample from NRW, the precursor band was weaker in density than that of the active form.

This shows that *A. sobria* can produce ALH in the medium supplemented with seawater; however, the processing of ALH does not proceed efficiently in culture with NSW.

Proteolytic and hemolytic activities of culture supernatant. The above result suggested that *A. sobria* can produce ALH in seawater as well as in river water; however, the ability of *A. sobria* to produce ASP is suppressed in seawater. In order to confirm the production and the non-production of these toxins in medium supplemented with different types of water, we measured the activities of protease and ALH of these samples.

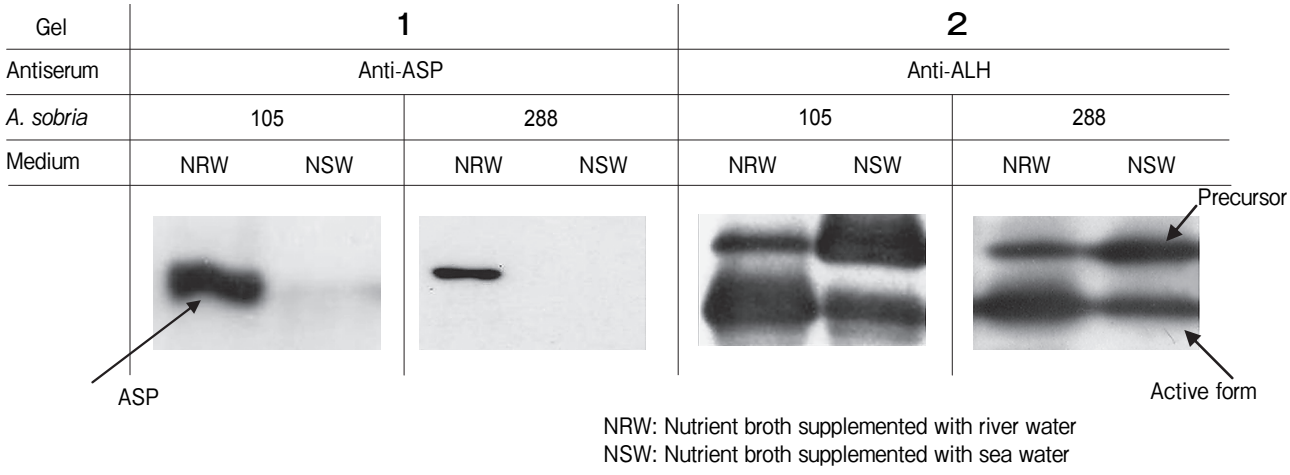
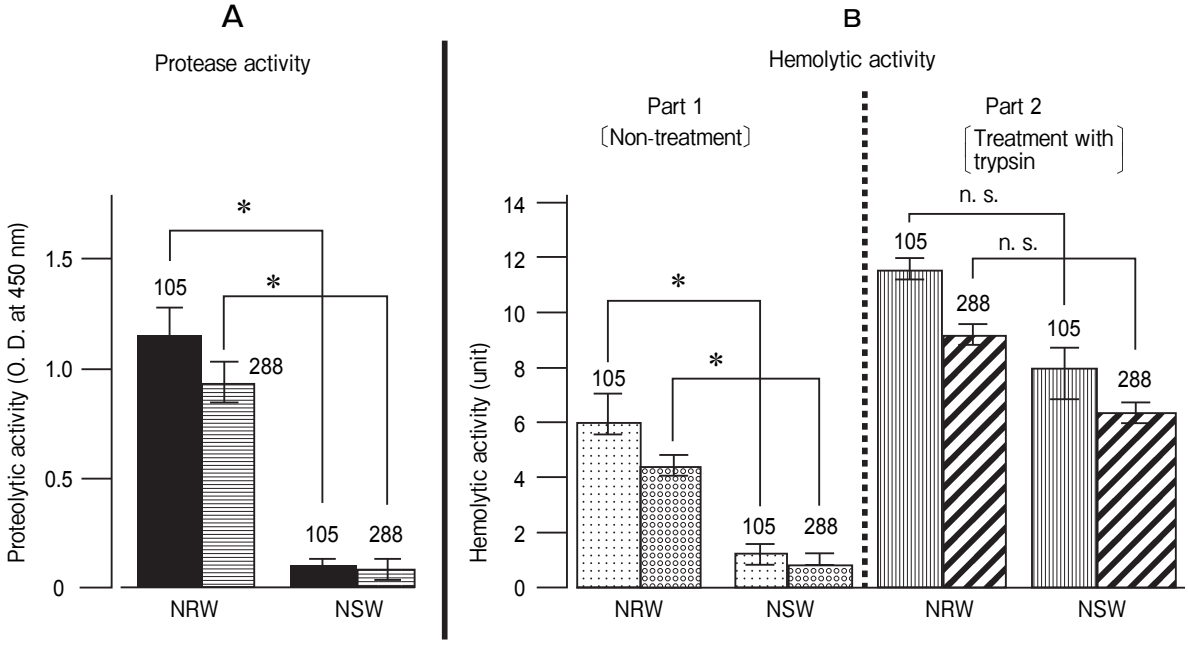


Fig. 2. Effect of environmental waters on production of serine protease (ASP) and aerolysin-like hemolysin (ALH) by *A. sobria* 105 and *A. sobria* 288. The cell suspensions obtained by 12-h cultures in NRW and NSW in the Fig. 1 experiments were centrifuged and their culture supernatants were recovered. The proteins in the supernatant were collected by treatment with TCA and separated by SDS-PAGE using gel with 16% acrylamide. After electrophoresis, the proteins were electrophoretically transferred onto nitrocellulose membranes and detected using antiserum against ALH (gel 2) and ASP (gel 1), and horseradish peroxidase-conjugated donkey anti-rabbit IgG according to the manufacturer's instructions.

Protease activity was measured by a caseinolytic assay as described in Materials and Methods. The proteolytic activity of the culture supernatant from NRW was high, but the activity of the sample from NSW was extremely low (Fig. 3A). This indicates that *A. sobria* is unable to produce proteases outside the cells in seawater.

The hemolytic activity of the samples was measured by the tube method as described in Materials and Methods. The sample from NRW presented high hemolytic activity. The activity of the sample from NSW was low, though some activity was detected (Fig. 3B, part 1).

The immunoblotting indicated the existence of the precursor of ALH in the sample from NSW. Then we treated the sample with trypsin to process these precursors and examined the hemolytic activity of the trypsinized sample. The trypsinized supernatant expressed higher hemolytic activity compared with the untreated sample (Fig. 3B, part 2). This indicated that the protoxin of hemolysin, which was converted to the active form by treatment with trypsin, was produced in the supernatant by the culture with NSW.



NRW: Nutrient broth supplemented with river water
NRW: Nutrient broth supplem ented with sea water
105: *A. sobria* 105
288: *A. sobria* 288

Fig. 3. Protease activity (**A**) and hemolytic activity (**B**) of the culture supernatants of *A. sobria* 105 and *A. sobria* 288. The cell suspensions obtained by 12-h cultures in NRW and NSW shown in Fig. 1 were centrifuged and their culture supernatants were recovered. **A**, The proteolytic activity of the culture supernatant was examined. Proteolytic activity was determined by azocasein assay [21]. The amount of azocasein digested by the proteases was measured by absorbance at 450 nm, and the measured values were used as the value expressing the proteolytic activity of the corresponding sample; **B**, The hemolytic activity of the culture supernatant was examined. A portion of the culture supernatant was treated with trypsin at 37℃ for 10 min as described in the text. These samples treated with trypsin are shown in part 2. As a control, the solvent used for making trypsin solution was added to the samples in part 1 (non-treatment). After incubation, the samples were diluted with 10 mM Tris-HCl buffer (pH 7.2) containing 0.9% NaCl. The hemolytic activity of the sample was determined as described in the text. The hemolysis of each sample was measured by absorbance of the solution at 540 nm. One hemolytic unit was defined as the amount of toxin causing 50% hemolysis. The hemolytic activity of the sample was expressed by the reciprocal of the dilution of the sample necessary to give one unit. The error bars indicate standard deviations of 2 experiments performed in triplicate. *, $p<0.001$; n. s., not significant. Numbers above the bars indicate strain's number.

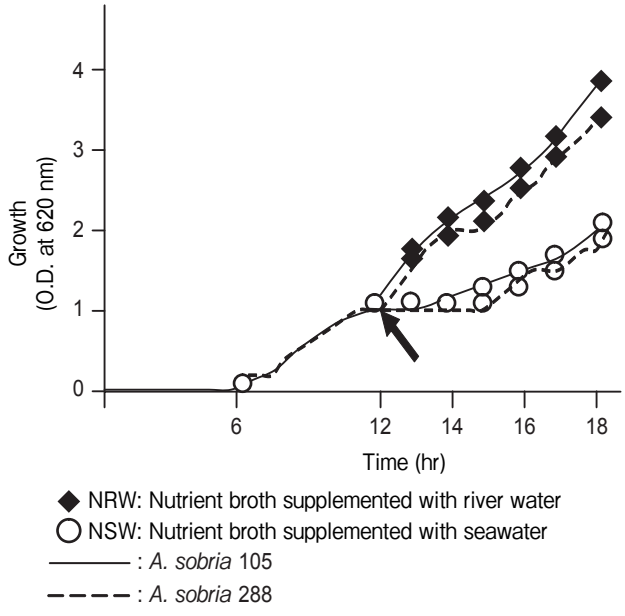


Fig. 4. Growth of *A. sobria* strains, which were grown in broth containing seawater, in new medium. *A. sobria* was cultured in 100 ml NSW with shaking at 37℃ for 12h. The bacterial pellet was collected by centrifugation and suspended in 2ml of distilled water. One ml was inoculated into 50 ml of NRW and another 1ml was inoculated into 50ml of NSW. The arrow in the figure indicates the position where the medium was changed. These were cultured at 37℃ for another 6h. Bacterial growth was monitored by the absorbance at 620nm.

Effect of change of water environment. From the above results, we thought that *A. sobria* was unable to produce ASP in NSW. However, it was likely that a mutant strain of *A. sobria* that could not produce ASP selectively grew in NSW. These mutant strains cannot produce ASP if they are transferred to new environment. To examine the generation of such mutant strains in NSW, we changed the medium from NSW to NRW in the middle of the culture, and examined the production of toxins in the new medium.

At first we cultured *A. sobria* in 100ml NSW with shaking at 37℃ for 12h. Then the bacterial pellet was collected by centrifugation and suspended in 2ml of distilled water. One ml was inoculated into 50ml of NRW and another 1ml was inoculated into 50ml of NSW. These were cultured at 37℃ for 6h. The bacteria suspended in NRW grew well while the growth of bacteria in NSW was much lower (Fig. 4).

The production of ASP and ALH was examined by the immunoblotting test and activity test. The results showed that these toxins were produced at a similar rate to those observed in Figs. 2 and 3 (data not shown). This showed that *A. sobria* grown in NSW retained the ability to produce ASP.

Discussion

This study showed that *A. sobria* does not produce ASP in medium supplemented with seawater; however, it does produce ALH in this medium. In the medium supplemented with river water, both toxins, ALH and ASP, were produced. This means that ALH is produced constitutively; however, the production of ASP is regulated by salt in the medium.

As shown in Fig. 1, the growth of *Aeromonas* is suppressed in NSW. This impressed to us that the amount of exotoxins produced by the culture in NSW was low compared with those from the culture in NRW. However, the repression of production of ALH in NSW was not significant (Fig. 3B, part 2). This means that *Aeromonas* did produce ALH in NSW (Fig. 3B, part 2). By contrast, the ASP activity of the sample obtained from NSW was extremely low (Fig. 3A). Since the bacteria did grow in NSW, albeit at a growth rate about half that in NRW (Fig. 1), such complete inhibition of production of ASP might be due to special regulation. At present, there are insufficient data to understand why the production of ASP is regulated by salt in the medium while the production of ALH is not regulated.

As described, *A. sobria* can produce the precursor, ALH, in seawater, and it has been shown that ALH can be activated by treatment with trypsin [30]. Therefore, ingestion of ALH could lead to the onset of the disease, because ALH could be activated by trypsin in the intestine.

ASP is the primary virulence factor of *Aeromonas*. It can induce vascular leakage and cause edema in skin. The action is responsible for the ulcerative syndrome induced by the infection of *Aeromonas*. *Aeromonas* was originally known as a pathogen of the ulcerative syndrome of fish. The damage caused by the infection tends to be restricted to freshwater fish; reports of damage in seawater fish are rare. The low incidence of *Aeromonas* infection in seawater fish may be linked to the non-production of protease by *Aeromonas* in seawater.

Our results indicate that salt in the aquatic envi-

ronment represses the production of protease(s) by *Aeromonas*. It seems that *Aeromonas* living in seawater do not produce ASP (Fig. 3A), though they retain the ability to produce ASP, and begin to produce ASP when the surrounding conditions are changed to be suitable for the production of ASP (Fig. 4). Thus, ASP might be responsible for furunculosis and ulcerative syndrome associated with *Aeromonas*. Therefore, if *Aeromonas* living in seawater, where they do not produce ASP, are moved to new, salt-free surroundings, they might produce ASP there, and induce much damage. In fact, *Aeromonas* were the most common cause of skin and soft tissue infections among the survivors of the 2004 Tsunami in Thailand [31, 32]. Infection with *Aeromonas* is often fatal. Special attention to *Aeromonas* infection should be taken after any water-associated injury or natural disaster.

Protease is an important requirement for bacteria to survive in environment. Some proteases released outside the cell digest proteins into small peptides, which bacteria can incorporate into cell. Without this digestion, bacteria cannot utilize proteins as nutrition. Some proteases function to process precursor proteins into their mature form [26]. As described, the protease activity of *Aeromonas* in salty conditions is low (Fig. 3). The depressed protease activity of *Aeromonas* in seawater may influence the livelihood and/or survivability of *Aeromonas* in seawater. The growth of *Aeromonas* in NSW is slow compared with that in NRW (Fig. 1), and the number of *Aeromonas* in seawater is low compared with that in river environments [7, 33]. Due to these factors, *Aeromonas* can harmoniously coexist with other bacteria in the natural world. If by some accident, *Aeromonas* living in seawater are moved to the land surface, this coexistence might be broken. In fact, in Hurricane Katrina in New Orleans, a large number of *Aeromonas* were isolated from flood water samples [34]. Our study may point the direction for further investigations of the coexistence of bacteria in the natural world. Further studies on the production of exotoxins in brackish or estuarine water might present valuable data not only on the existence of *Aeromonas* in the natural world but also on the role of exotoxins in the survival of bacteria.

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